Cholinergic-NO-cGMP mediation of sildenafil-induced antinociception

Chandrashekhar S Patil, Naveen K Jain, Vijay Pal Singh, Shrinivas K Kulkarni

Pharmacology Division, University Institute of Pharmaceutical Sciences Panjab University, Chandigarh 160 014, India
R&D Division, Panacea Biotec Ltd., Chandigarh Road, Lalru, 140 501, India

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Acetylcholine and cholinomimetic agents with predominant muscarinic action are known to increase the concentration of cGMP by activation of nitric oxide signaling pathway in the nociceptive conditions. The present study was aimed to investigate the NO-cGMP-PDE5 pathway in nociceptive conditions in the experimental animals. Nociceptive threshold was assessed by acetic acid-induced writhing assay (chemo nociception) or carrageenan-induced hyperalgesia. Sildenafil [1-5 mg/kg, ip, 50-200 μg/paw, intraplantar (ipl)] produced dose dependent antinociception in both the tested models. Co-administration of acetylcholine (50 mcg/paw, ipl) or cholinomimetic agent, neostigmine (0.1 mg/kg, ip and 25 mg/paw, ipl) augmented the peripheral antinociceptive effect of sildenafil. This effect was sensitive to blockade by L-NAME (20 mg/kg, ip, 100 μg/paw, ipl), a non-selective NOS inhibitor and methylene blue (1 mg/kg, ip), a guanylate cyclase inhibitor, which per se had little or no effect in both the models of nociception. Further, the per se analgesic effect of acetylcholine and neostigmine was blocked by both L-NAME and methylene blue in the models of nociception, suggesting the activation of NO-cGMP pathway. Also, both L-NAME and methylene blue blocked the per se analgesic effect of sildenafil. These results indicate the peripheral accumulation of cGMP may be responsible for antinociceptive effect, and a possible interaction between cholinergic agents and PDE5 system in models of nociception.

Keywords: PDE 5 inhibitors, Cholinergic, Cholinomimetic agents; NOS, Nociception
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Presence of intrinsic cholinergic inhibitory pathways in modulating pain has been demonstrated as cholinomimetic agents administered centrally or peripherally are known to inhibit nociception by increasing the endogenous acetylcholine. A role for cholinergic system, both central and peripheral, in elicitation of antinociceptive response has been ascribed experimentally as well as clinically1-3. Centrally it appears to modulate this effect through nitric oxide synthase (NOS) pathway via enhanced acetylcholine as NOS inhibitor antagonized the antinociceptive response of cholinomimetics. A peripheral analgesic effect of cholinergic agents, as dibutyl cyclic GMP mimicked acetylcholine induced analgesia, suggesting that cholinergic agents may cause analgesia by altering cyclic GMP at the nociceptor level1.

The role of NO in the nociception and antinociception is still controversial. The early up-regulation of NOS in the spinal cord and increased production of spinal NO after nerve constriction and tissue inflammation4-11 during acute and chronic pain favours a pronociceptive action of NO. In contrast, recent studies where systemic administration of NO precursor, L-arginine has been shown to produce analgesia in mice12,13. Besides, peripheral activation of NO-cGMP pathway in antinociception in animal models14 provide supportive evidence that spinal NO plays a role in antinociception.

Phosphodiesterases (PDEs) are the large group of structurally related enzymes that catalyse the hydrolysis of 3' 5'-cyclic nucleotides to corresponding inactive nucleosides 5'monophosphate. Eleven families (PDE 1-11) of PDEs have been identified with each family being the product of a separate gene and usually comprise several isoforms15. PDE5 is predominant cGMP-specific PDE expressed in skeletal, cardiac and smooth muscle16 and is responsible for the hydrolysis of cGMP16,17. Sildenafil is a potent selective and reversible phosphodiesterase 5 inhibitor15 that blocks cyclic GMP hydrolysis (ki = 3 nM). Recently Jain et al.14, have reported the peripheral analgesic effect of sildenafil in the animal models of nociception on systemic and local administration. This effect was found to be mediated through inhibition of cyclic GMP specific phosphodiesterase type 5 (PDE5). In the present study the possible interaction between

*Correspondent author
Phone: +91-172-534114
Fax: +91-172-541142
E-mail: skpu@yahoo.com
PDE5 and cholinergic system was explored in the animal models of nociception and the possible participation of NO-cGMP-PDE5 pathway.

**Materials and Methods**

**Animals**—Albino mice (Swiss strain, 20-30 g) and rats (Wistar strain, 150-200 g) of either sex (Central Animal House, Panjab University, Chandigarh, India) were housed under standard laboratory conditions and kept under a 12:12 hr L:D cycle. Experiments were carried out between 0900 and 1800 hrs. All the experimental protocols were approved by the Institutional Animal Ethics Committee.

**Antinociceptive study—Writhing test (acetic acid assay)—**A 1% acetic acid solution (10 ml/kg, ip) was used to produce writhing in mice. The number of wriths (constriction of abdomen, turning of the trunk (twist) and extension of the hind limbs) due to acetic acid was expressed as the pain response. The number of wriths per animal was counted during a 20 min session, beginning 3 min after the injection of acetic acid.

**Induction and assessment of carrageenan-induced hyperalgesia—**Acute inflammation was induced in the right hind paw by injecting, ip, 0.1 ml of freshly prepared solution of 1% carrageenan. The left paw received 0.1 ml of saline which served as control. The response to inflammatory pain was determined by measuring the mechanical nociceptive pressure by the paw pressure test (Ugo Basile, Italy). The apparatus was set up to apply a force of 0-1000 g, increasing from zero. The nociceptive threshold was taken as the end point at which the rat vocalized or struggled vigorously.

The test drug sildenafil and acetylcholine and/or neostigmine were injected in the volume of 50 μl intraplantarly in right hind paw 30 min prior to the carrageenan challenge and the pressure threshold was observed at 15, 30, 60, and 120 min. The time selection was made on the basis of the preliminary studies.

**Locomotor activity test—**The animal locomotor behavioural pattern was monitored using activity meter (IMCORP, India). The animals were individually placed in a plexiglass cage (40×15×15 cm) and the total activity count registered for 5 min. The locomotor activity was expressed in terms of total photo beams counts/5 min/animal.

**Drugs—**Sildenafil citrate (Panacea Biotec. Ltd), L-NAME and carrageenan IV (Sigma, U.S.A.), methylene blue, acetylcholine chloride and acetic acid (S D Fine Chemicals), neostigmine (Neon Labs, Mumbai) were obtained commercially.

**Statistical analysis—**Results are expressed as mean ± S E. The significance of the difference in the responses of treatment groups in comparison to the control was determined by one-way analysis of variance (ANOVA) followed by Dunnett’s test. A level of \( P < 0.05 \) was considered statistically significant.

**Results**

**Antinociceptive activity of sildenafil in mice and rats—**Systemic administration of sildenafil (1-5 mg/kg, ip) showed a dose-dependent decrease in the number of writhes indicating an increase in pain threshold (Fig. 1a). In carrageenan-induced hyperalgesia ipsilateral, but not contralateral administration of sildenafil (50-200 mg/paw, ip) exhibited a dose-dependent increase in pain threshold (Fig. 2). The antinociceptive effect of sildenafil (1 mg/kg, ip and 50 μg/paw, ip) was blocked by L-NAME (20 mg/kg, ip, 100 μg/paw; ipl) and methylene blue (1 mg/kg, ip) in both the tests of nociception (Fig. 3).

**Antinociceptive activity of acetylcholine and/or neostigmine in mice and rats—**Neostigmine (0.1-0.5 μg/kg, ip) produced a dose-dependent increase in nociceptive threshold in writhing assay in mice (Fig. 1b). Acetylcholine (50-200 μg/paw, ipl) and neostigmine (25-100 ng/paw) produced a dose-dependent antinociceptive effect against carrageenan-induced hyperalgesia in rats (Fig. 4). The antinociceptive effect of acetylcholine (50 μg/paw, ipl) was blocked by L-NAME (100 μg/paw, ipl) and methylene blue (1 mg/kg, ip) in carrageenan-induced hyperalgesia. Also, the antinociceptive effect of neostigmine (0.1 μg/kg, ip and 25 mg/paw, ipl) was blocked by L-NAME (20 mg/kg, ip, 100 μg/paw, ipl) and methylene blue (1 mg/kg, ip) in both the tests of nociception (Fig. 7).

**Effect of acetylcholine and/or neostigmine on sildenafil-induced antinociception in mice and rats—**The lowest effective dose of acetylcholine (50 mcg/paw, ipl) potentiated the antinociceptive effect of sildenafil (50 mcg/paw, ipl) in carrageenan-induced hyperalgesia and the effect was blocked by L-NAME (100 mcg/paw, ipl) and methylene blue (1 mg/kg, ip) (Fig. 5). Similarly, neostigmine (0.1 μg/kg, ip, and 25 mg/paw, ipl) enhanced the antinociceptive effect of sildenafil (1 mg/kg, ip, 50 μg/paw, ipl) in both the tests of nociception and the effect was blocked by L-NAME (20 mg/kg, ip, 100 μg/paw, ipl) and methylene blue (1 mg/kg, ip) (Figs 1c and 6).
Fig. 1—Antinociceptive effect of (A) sildenafil (1-5 mg/kg, ip), (B) neostigmine (0.1-0.5 mcg/kg, ip), and (C) their combination and its reversal by L-NAME (20 mg/kg, ip) and methylene blue (1 mg/kg, ip) in the mouse writhing test. Data are expressed as mean ± S.E. (n = 6 or 8 mice per group). *P < 0.05 as compared with control, **P < 0.05 as compared with sildenafil (1 mg/kg, ip) and neostigmine (0.1 mg/kg, ip) per se, ***P < 0.05 as compared with sildenafil and neostigmine combination.

Fig. 2—Effect of sildenafil (50-200 μg/paw, i.pl) against carrageenan-induced hyperalgesia (paw pressure test) in rats. Data are expressed as mean ± S.E. (n = 6 or 8 rats per group). *P < 0.05 as compared with control, **P < 0.05 as compared with carrageenan treated group.

Fig. 3—Effect of L-NAME (20 mg/kg, ip) and methylene blue (1 mg/kg, ip) on sildenafil (1 mg/kg, ip) and neostigmine (0.1 μg/kg, i.p.) in the mouse writhing test. Data are expressed as mean ± S.E. (n = 6 or 8 mice per group). *P < 0.05 as compared with control. **P < 0.05 as compared with sildenafil (1 mg/kg, ip) and neostigmine (0.1 mg/kg, ip) per se.

Effect of neostigmine, sildenafil and their combinations on locomotor activity in mice—No significant behavioural or motor dysfunctions were observed on treatment of sildenafil (1-5 mg/kg, ip), neostigmine...
(0.1-0.5 \mu g/kg, ip), or their combination in mice (Table 1).

Discussion

Antinociceptive action of sildenafil—Findings of present study indicate that sildenafil on systemic (1-5 mg/kg, ip) and local (50-100 \mu g/paw) administration produced dose-dependent antinociceptive effect in animal models of nociception. This effect appears to be a local action, as the contralateral administration of the drug was ineffective to reduce nociception. This is suggestive of a major contribution by peripheral PDE 5 and NO-cGMP pathways in sildenafil-induced peripheral antinociception. The sildenafil produces antinociception probably through the inhibition of cyclic GMP degradation by PDE 5 enzyme, suggesting the major participation of peripheral PDE 5 and NO-cGMP pathways.

Fig. 4—Effect of (A) acetylcholine (50-200 \mu g/paw, ip) and (B) neostigmine (25-100 \mu g/paw, ip) against carrageenan-induced hyperalgesia (paw pressure test) in rats. Data are expressed as mean \pm S.E. (n = 6 or 8 rats per group). *P < 0.05 as compared with control, "P < 0.05 as compared with carrageenan treated group.

Fig. 5—Effect of acetylcholine (ACH: 50 \mu g/paw, ip) on sildenafil (SIL: 50 \mu g/paw, ip)-induced antinociception and its modification by L-NAME (100 \mu g/paw, ip) and methylene blue (1 mg/kg, ip) in carrageenan-induced hyperalgesia (paw pressure test) in rats. Data are expressed as mean \pm S.E. (n = 6 or 8 rats per group). *P < 0.05 as compared with control, "P < 0.05 as compared with carrageenan treated group, "P < 0.05 as compared with sildenafil (50 \mu g/paw) and acetylcholine (50 \mu g/paw) per se, "P < 0.05 as compared with sildenafil and acetylcholine combination.

Fig. 6—Effect of neostigmine (NEO: 25 \mu g/paw, ip) on sildenafil (SIL: 50 \mu g/paw, ip)-induced antinociception and its modification by L-NAME (100 \mu g/paw, ip) and methylene blue (1 mg/kg, ip) in carrageenan-induced hyperalgesia (paw pressure test) in rats. Data are expressed as mean \pm S.E. (n = 6 or 8 rats per group). *P < 0.05 as compared with control, "P < 0.05 as compared with carrageenan treated group, "P < 0.05 as compared with sildenafil (50 \mu g/paw) and neostigmine (25 \mu g/paw) per se, "P < 0.05 as compared with sildenafil and neostigmine combination.
It has been postulated that the administration of anticholinesterase neostigmine, modulated central and peripheral sites of intrinsic cholinergic inhibitory pathway of pain perception. This involves hyperpolarization of neurons, reduction in the release of pronociceptive neurotransmitters and activation of the NO-cGMP pathway mediate cholinergic antinociception by elevating endogenous acetylcholine.

In the present study acetylcholine (50-200 μg/paw, ip) and neostigmine (0.1 μg/kg, ip and 25 –100 ng/paw, ip) produced a dose-dependent analgesic effect in models of nociception. This effect may be due to spinal or peripheral accumulation of acetylcholine as observed by Duarte et al. Further, the antagonism of the effects of acetylcholine and neostigmine by L-NAME and methylene blue indicate a role for NO-cGMP pathway.

This is supported by the observation that potentiated response was recorded when lowest effective dose of sildenafil and cholinomimetic agents was used. Additionally, this enhancement was reversed by...
L-NAME and MB. These data favour the contention of role for nitric oxide synthase in the antinociceptive response of acetylcholine. A hypothetical schematic diagram is present in Fig. 8. A similar role in acetylcholine-induced vasodilation has been reported\(^7\). The possibility that the drugs used in the present study are known to cause hypotension by increasing the levels of cGMP\(^8\) thereby may alter the ability of the animals to respond to nociceptive stimuli is unlikely as, acetylcholine and neostigmine per se and in combination with sildenafil neither produced a significant change in blood pressure (data not shown) nor altered its locomotion. Besides other classical cholinergic symptoms viz. lacrimation and salivation was observed at the tested doses. Considering together, the findings of the present study clearly defines a significant role for NO and cGMP in cholinergic mechanisms involved in the elicitation of antinociception.

In summary, the current study provides the evidence that sildenafil and cholinergic/cholinomimetic agents produced antinociceptive effect in the models of nociception. In addition sildenafil increased the antinociceptive effect of cholinergic/cholinomimetic agents, which may be due to the peripheral accumulation of cGMP and a possible interaction between cholinergic agents and PDE 5 system.

References

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